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Incidence of Virulent Factors in Staphylococci Isolated from Clinical and Foods Specimens in Egypt

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ONE HUNDRED Gram positive staphylococci isolates were isolated from 80 clinical specimens and 20 food samples using Baird Parker and mannitol salt agar media. Sixty isolates out of 80 (75%) isolated from clinical specimens were found to be coagulase positive staphylococci while the other 20 isolates (25%) were proved to be coagulase negative staphylococci. Meanwhile, all staphylococci isolated from food samples (20 isolates) were found to be coagulase negative isolates. The antibiotic susceptibility profile of all isolated staphylococci against 11 antibiotics indicated that 8 isolates were found to be resistant to more than 4 antibiotics which means that they are multi-drug resistant (MDR). Following the key of Bergey's Manual of Determinative Bacteriology, the 2 coagulase positive clinical isolates (41 and 66) were preliminary identified as *S. aureus* and the other 6 coagulase negative staphylococci isolates were found to be related to different species of *Staphylococcus* genus. Using specific designed primers, some toxin target genes namely: *ent*A, *entC*, *entD1* and *hlg* were screened in the 8 selected MDR isolates. The isolates encoded 7 and 11, preliminary identified as *S. saprophyticus* 7 and *S. xylosus* 11, showed obvious significant amplicons of toxins genes *entA*, *entD1* and *hlg* while the clinical isolates 41 and 66 which were preliminary identified as *S. aureus,* possessed *entA* and *hlg* genes only. Identification of coagulase negative *S. xylosus* 11 containing more than 3 toxin genes was confirmed by amplification of 16S rRNA gene which showed 99% similarity with *S. xylosus* strain. This sequence was deposited in Genbank under accession number MH118574.

Keywords: Genus *Staphylococcus*, Virulence factors, Pathogenicity, Multidrug resistance (MDR), Enterotoxins genes.

Introduction

Coagulase-negative staphylococci (CNS) are the most frequent constituent of the normal flora of components of various parts of the skin and of the respiratory and gastrointestinal system mucosa of human; they may also appear in animals and foods tuff (Longauerova, 2006 and Becker et al., 2014). These organisms are common contaminants in clinical specimens as well as increasingly recognized as agents of clinically significant infection, including bacteremia and endocarditis (Chu et al., 2008).

Staphylococcus enterotoxins are relatively resistant to heat and to the proteolytic enzymes trypsin, pepsin and renin, which enables their passage through the gastrointestinal tract without losing activity (Huy, 1994). At least thirty four different extracellular proteins are produced by pathogenic *Staphylococcus* strains, and several of them already play a definite role in the pathogenesis of recognized staphylococcal diseases (Lisa, 2004 and Vianello, 2006).

Virulence and pathogenicity had been commonly related to coagulase positive *S. aureus* species as common clinical and foodborne pathogens. Enterotoxins are mainly produced by coagulase positive staphylococci (Le Loir et al., 2003 and Chiang et al., 2008). Meanwhile, some coagulase-negative staphylococci (CNS) are reported to be involved in a variety of human and animal infections (Kloos & Bannerman, 1995). CNS can contaminate foods because humans are common carriers of these microorganisms and some may be related to specific human infections (Bergdoll, 1995).

Some *S. aureus* strains and coagulase-negative staphylococci secreted staphylococcal virulence factors as staphylococcal enterotoxins (SEs) and toxic shock syndrome toxin 1 (TSST-1) (Novick et al., 2001 and Cunha & Calsolari, 2008). Klotz et

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al. (2003) detected *S. aureus* enterotoxins A, B, C1, and D genes which encoding as SEA (*entA*), SEB (*entB*), SEE (*entE*) and SED (*entD*) by using real-time fluorescence PCR assay. Also, *Staphylococci* produces five different membrane damaging toxins, four hemolysins (alpha-, beta-, gamma- and delta-hemolysin) and leukocidin (Koneman, 1997). The alpha-hemolysin encodes *hla* is dermonecrotic and neurotoxic and can be lethal in a variety of animal systems contributing role in formation of abscesses (Gray & Kehoe, 1984). Gamma-hemolysin toxins are characterized by two non-associated secreted proteins, referred to as S and F components. The gamma toxin locus expresses two class S components (HlgA and HlgC) and one class F component (HlgB) (Supersac et al., 1998).

The recognition of CNS as etiological agents may be due to the valorization of this group of organisms as opportunistic pathogens and to the increasing use of invasive procedures such as intravascular catheters and to prosthetic interference (da Cunha et al., 2006). Evidence indicates that pathogenicity might be related to the production of an extracellular polysaccharide, known as slime that permits these microorganisms to adhere to smooth plastic surfaces, colonizing catheters, prosthetic heart valves, pacemakers, and joint prostheses (Vogel et al., 2000). According to Koneman et al. (1997), CNS produce other virulence factors, such as hemolysins, lipases, proteases, and toxins.

Recent reports of the involvement of some coagulase negative staphylococci in a variety of human and animal infections have raised interest (Gillespie et al., 2009 and Becker et al., 2014). El-Jakee et al. (2013) reported that CNS have emerged to be pathogens causing intramammary infections in Egyptian dairy herds. The current study was conducted to investigate the incidence of coagulase negative and positive staphylococci isolated from clinical and food samples and study their virulence and antibiotic susceptibility profile.

Materials and Methods

Samples collection

This study was conducted on eighty clinical specimens of pus, sputum and urine collected from eighty patients admitted to Zagazig University Hospital, Zagazig, Egypt in the period from January to March, 2014. Also, the study was conducted on twenty isolates from different sources of food (meat and meat products; milk and milk products) which collected from Sharkia Markets in same the period. The specimens were collected and transported according to Murray et al. (2007).

Isolation, purification and identification of bacterial isolates

Swabs of clinical specimens were directly streaked on agar surface of specific media plates, meanwhile 10g of food samples were homogenized and suspended in 90ml sterile peptone water and incubated at 37°C for 3h. Further on, loopful inocula of prepared food samples were streaked on surface of agar specific media; Baird Parker agar (Baird-Parker et al., 1969) and Mannitol salt agar (Merlino et al., 1996). Plates were incubated at 37°C for 24h.

Morphological and physiological characteristics of the selected isolates were studied. Media and reagent were prepared according to standard procedures as described by Lennette et al. (1979). The selected bacterial isolates were preliminary identified according to Holt et al. (1994).

Identification of selected isolate was confirmed by sequencing of partially amplified 16S rRNA gene. The DNA was extracted from bacteria following the protocol recommended by Sambrook & Russell (2001). 16S rRNA gene was sequenced using 5'-AGAGTTTGATCC TGGCTCAG-3' as forward primer and 5'-GGTTACCTTGTTACGACTT-3' as reverse primer. NCBI BLAST program (www.ncbi.nlm.gov/blast) and ClastalW2 program (https://www.ebi.ac.uk/Tools/msa/clustalw2/) for sequence similarity and phylogenetic analyses was used to assess the similarities of the obtained 16S rRNA gene sequence in Genbank database.

Antibiotic susceptibility test

Susceptibility of the tested bacterial isolates to eleven antibiotics (vancomycin, amikacin, mipenem, ampicillin, cefotaxime, norfloxacin, ciprofloxacin, ofloxacin, gentamicin, tetracycline and ampicillin/sulbactam (unasyn) was determined using disk diffusion technique according to NCCLS (1999). These antibiotics were selected depending on their different groups and their action in Gram positive bacteria.

Determination of virulence factors produced by selected isolates

The selected MDR isolates resistant to more than 4 antibiotics were screened for their capability to produce specific virulence factors, namely, hemolysin, protease and lecithinase enzymes on

blood agar, casein agar and egg yolk agar media, respectively, at 35°C for 24h using agar well diffusion assay according to Klaenhammer (1988). Also, coagulase (Chessbrough 2000); gelatinase (Collee & Marr, 1996), fibrinolytic activities (Christie & Wilson, 1941) and biofilm formation (Mathur et al., 2006) by selected isolates were tested.

Screening of the selected strains for target toxin genes using conventional polymerase chain reaction (PCR) technique

DNA templates of the tested bacterial cultures were prepared according to the method described by Osek (2000). Four pairs of primers specific for genes; *ent*A, *ent*D1, *hl*g and *ent*C1 were designed as shown in Table 1 and described previously by Ye et al. (2012).

All the reactions for PCR analyses were carried out using 0.25µl (1.25 U) Taq DNA polymerase (Thermoscientific, USA). A total reaction volume of 25µl was used, including 2.5µl of 10x PCR reaction buffer, 0.5µl dNTPs mixture (100µM), 0.5µl of each primer (10pmol), and 2µl template DNA were added. PCR amplification was carried out using a Master Cycler Gradient PCR. In all cases, The PCR program was 2min at 95°C initial denaturation step followed by 35 cycles (30sec at 95˚C denaturation, 30sec primer annealing step at 56˚C and 1min extension period at 72°C). PCR products were visualized on a 1% (w/v) agarose gel containing (2µl) ethidium bromide as described by Sambrook et al. (1989). 1x Tris-acetate-EDTA (TAE) buffer was used for gel preparation and as running buffer. The expected amplicons was visualized using UV- transilluminator and Gel doc 100 systems (Biorad, Germany). 1Kbp DNA plus ladder was used to determine the molecular size of the resulted amplicons.

Sequencing of amplified genes

Sequencing of PCR products was performed for all amplified genes at GATC Company using ABI3730xl DNA sequencer system. All PCR products were firstly column purified using PCR product purification kit (Qiagen, Germany). Toxins genes (*ent*A, *ent*C1, *ent*D1 and *hlg*) were sequenced using the designed forward primers listed in Table 1.

Results and Discussion

Members of the *Staphylococcus* genus, especially *Staphylococcus aureus*, are the most common pathogens found in hospitals and in community-acquired infections (Vaidieki et al., 2007). Some of their pathogenicity is associated with enzyme and toxin production (Casadevall & Pirofski, 2009). *S. aureus* was the most studied species in the genus. During the last decade, increased infections caused by coagulase-negative staphylococci (CNS) have been reported (Gillespie et al., 2009). Therefore, further studies on virulence factors that have not yet been completely elucidated in order to characterize the pathogenic potential of this group of bacteria. Several staphylococcal species produce enterotoxins; a family of related proteins responsible for many diseases, such as the toxic-shock syndrome, septicemia and food poisoning. To date, 23 different enterotoxin types have been identified besides to TSST-1, and they can be divided into five phylogenetic groups (Vasconcelos & da Cunha, 2010). The mechanism of action of these toxins includes super antigen activity and emetic properties, which can lead to biological effects of infection (Vasconcelos & da Cunha, 2010). Various reports have described the isolation of CNS and the association of these bacteria with clinically significant diseases (Azih & Enabulele, 2013).

TABLE 1. Primer sequences and molecular sizes of the expected amplicons of target the six tested enterotoxin genes.

Target gene (s)	Primer name	Primer sequence	Product size (bps)
Enterotoxin A (*entA*)	entA-FW	5'-TCA TTG CCC TAA CGT TGA CAA-3'	774
	entA-Rev	5'-CCT CTG AAC CTT CCC ATC AA-3'	
Enterotoxin D1 (*entD1*)	entD1-FW	5'-CCG CGC TAA ATA ATA TGA AAC A-3'	777
	entD1-Rev	5'-AAA TAG CGC CTT GCT TGT G-3'	
Gamma hemolysin (*hlg*)	hlg-Fw	5'-AGA AGA TAT CGG CCA AGG TG-3'	1200
	hlg-Rev	5'-TCA ACG GCT AAA CGA TGT CT-3'	
Enterotoxin C1 (*entC1*)	entC1-FW	5'-AGA GAG CCA ACC AGA CCC TA-3'	801
	entC1-Rev	5'-CCA TTC TTT GTT GTA AGG TGG A-3'	

In the current study one hundred Gram positive staphylococci isolates were isolated from 80 clinical specimens (pus, sputum and urine) and 20 food samples (raw meat, raw poultry meat, raw milk, beef and cheese) using Baird Parker and mannitol salt agar media. Sixty isolates out of eighty (75%) were isolated from clinical specimens and were found to be coagulase positive staphylococci while the other 20 isolates (25%) were proved to be coagulase negative staphylococci. Meanwhile, all staphylococci isolated from food samples (20 isolates) were found to be coagulase negative isolates. All isolates were Gram-positive cocci in cluster arrangement, non-motile, nonsporulated and catalase positive (Data not shown). *S. aureus* is an extraordinary versatile pathogen, and it can cause a large spectrum of infections, from mild to severe and fatal (Lowy, 1998). *S. aureus* is known as one of the most frequent pathogens in both community and nosocomial infections, and it can cause septicemia, endocarditis, osteomyelitis, abscesses, pneumonia, wound infections, impetigo, cutaneous rash, in addition to various toxin mediated diseases (Foster, 2004 and 2005). The variety of such spectrum of clinical manifestations is mostly dependent on the numerous virulence factors produced by each strain (Le Loir et al., 2003). *S. aureus* is one of the most common clinical and food borne pathogens. The infections of *S. aureus* can be acquired through both hospital and community settings including food poisoning (Kadariya et al., 2014). El-Jakee et al. (2013) studied the prevalence and isolation of CNS from the examined subclinical mastitic cattle, buffaloes, sheep and goats with percentages of 16.6%, 59.4%, 50% and 55.6%, respectively.

Coagulase-negative staphylococci (CNS) are a diverse group of commensals inhabiting the skin and mucous membranes of humans and animals. However some species of CNS are known as important opportunistic human pathogens. The role of CNS as animal and human pathogens is less understood. Recently, interest in (CNS), had increased due to their increasing importance in hospital infection, particularly in nosocomial bacteremia (Campoccia et al., 2006) and food poisoning outbreaks (Guimarães et al., 2013 and Tarekgne et al., 2015).

The potential threat of antibiotic resistance in food and healthcare associated bacteria is a concern for public health. The antibiotic susceptibility profile of all isolated staphylococci (100 isolates) against 11 antibiotics, indicated that 8 isolates (2 coagulase and 6 non coagulase staphylococci) were multi-drug resistant (MDR) to more than 4 antibiotics. The clinical isolates showed high vancomycin susceptibility (98.75%) followed by amikacin, and imipenem with 92.5%, and 86.25% susceptibility, respectively (Table 2). On the other hand, 73.75% of total tested bacterial isolates were resistant to ampicillin while 23.75%, 22.5% and 21.25% were resistant to cefotaxime, norfloxacin and ciprofloxacin, respectively. Regarding food isolates, vancomycin, imipenem, norfloxacin, ofloxacin, amikacin, gentamicin, and ciprofloacin were highly effective with susceptibility percentage (100%) followed by tetracycline with 80%, meanwhile, 70% of tested bacteria were resistant to ampicillin. Similarly, Gundogan & Ataol (2013) studied the antimicrobial susceptibility profiles of 209 saphylococci and their results revealed high percentage of resistance for ampicillin (33.8%), tetracycline (26.3%), erythromycin (20.6%), methicillin (17.2%) and gentamicin (12.4%). Meanwhile, susceptibility to vancomycin, chloramphenicol, amikacin and clindamycin were 100% for all isolates. Also, El-Zawahry et al. (2013) reported that the most effective antibiotic for clinical bacterial isolates was amikacin (80%) followed by nitrofurantoin and norfloxacin with susceptibility percentage, 76.25% and 71.25%, respectively. Reda et al. (2017) reported that gentamycin (76%) was the most effective antibiotic against clinical bacterial isolates followed by amikacin and nitrofurantoin. Nunes et al. (2015) reported that the CNS strains isolated from commercial and artisanal salami showed multi-resistance to several antimicrobials of therapeutic importance in both human and veterinarian medicine, such as β-lactams, vancomycin, and linezolid. Some species of coagulase negative staphylococci can present health risks, since they have shown resistance to antibiotics of therapeutic importance, such as beta lactams. Due to the intensive and indiscriminate use of antibiotics for human and veterinarian therapeutic purposes, multi-resistant staphylococci strains might be selected when introduced in food matrices acting as reservoir of multi-resistant microorganisms that can spread by the consumption of an apparently safe food. This opinion has been emphasized by several authors (Babic et al., 2011 and Nunes et al., 2015).

TABLE 2. Comparative susceptibility of all bacterial isolates from clinical and food sources against different antibiotics.

Isolate source	Antibiotic	Symbol	Conc. µg/ disc	Resistant (R)		Intermediate (I)		Susceptible (S)	
				No.	%	No.	%	No.	%
Clinical	Vancomycin	VA	30	0	0	1	1.25	79	98.75
	Imipenem	IMP	10	7	8.75	4	5.00	69	86.25
	Norfloxacin	NOR	10	18	22.50	4	5.00	58	72.50
	Cefotaxime	CTX	30	19	23.75	11	13.75	50	62.50
	Ofloxacin	OFX	5	13	16.25	2	2.50	65	81.25
	Amikacin	AK	30	1	1.25	5	6.25	74	92.5
	Ampicillin	AM	10	59	73.75	0	0	21	26.25
	Tetracyclin	TE	30	16	20.00	1	1.25	63	78.75
	Gentamicin	CN	10	12	15.00	3	3.75	65	81.25
	Ciprofloxacin	CIP	5	17	21.25	1	1.25	63	78.75
	Ampicillin\ sulbactam(unasyn)	SAM	20	8	10.00	7	8.75	65	81.25
Food	Vancomycin	VA	30	0	0	0	0	20	100
	Imipenem	IMP	10	0	0	0	0	20	100
	Norfloxacin	NOR	10	0	0	0	0	20	100
	Cefotaxime	CTX	30	8	40	0	0	12	60
	Ofloxacin	OFX	5	0	0	0	0	20	100
	Amikacin	AK	30	0	0	0	0	20	100
	Ampicillin	AM	10	14	70	0	0	6	30
	Tetracyclin	TE	30	4	20	0	0	16	80
	Gentamicin	CN	10	0	0	0	0	20	100
	Ciprofloxacin	CIP	5	0	0	0	0	20	100
	Ampicillin\ sulbactam(unasyn)	SAM	20	5	25	0	0	15	75

The virulence factors of microorganisms in the *Staphylococcus* genus include surface components, such as the capsule, peptidoglycans, teichoic acid, protein A, collagen cell attachment protein, enzymes such as lipases, esterases, fatty-acid modifying enzymes, various proteases, hyaluronidase, hydrolytic enzymes, desoxirribonucleases, coagulase, catalase, betalactamase, staphylokinase, and various toxins, such as exfoliative toxin A and B, leukocidins, enterotoxins, TSST-1 and alpha, beta, gamma and delta hemolysins (Cunha et al., 2006). Staphylococcal toxins can be divided into two groups according to their capacity for lysing cells: Hemolysins or cytotoxins, which are capable of producing lesion directly to the outer membrane of target cells (Freer & Arbuthnott, 1982), and the so-called superantigen toxins, which do not present direct lytic action, but can produce lesions through the overproduction of cytotoxins from activated T-cells and from monocytes/macrophages (Herman, 1991).

In the present study, eight isolates encoded 41, 60, 61, 66, 67, 69, 7 and 11 were selected as multi-drug resistant (resistant to 4 or more antibiotics) and examined for virulence factors and the presence of target enterotoxins genes (*ent*A, *ent*D_1, *hl*g, *ent*C_1). Results in Table 3 revealed apparent lecithinase activity in 7 isolates out of 8 (87.5%), hemolytic activity and biofilm formation in 5 isolates (62.5%). Meanwhile, amylase and fibrinolytic activities were detected in 2 isolates only (25%) and gelatinase activity was completely absent in all selected MDR isolates. According to Holt et al. (1994), the 2 coagulase positive clinical isolates (encoded as 41& 66) were preliminary identified as *S. aureus* and the other 6 non coagulase staphylococci were found to be related to different species of *Staphylococcus* genus; *S. saprophyticus* 7, *S. xylosus* 11, *S. epidermidis* 60 and *Staphylococcus* sp. 67 and 69. In this connection, Gundogan & Ataol (2013) reported that 175 out of 209 total Staphylococci (83.7%) isolated from raw calf meat (minced), chicken drumsticks, raw milk, ice cream and white cheese samples were coagulase negative staphylococci and 34 (16.3%) were coagulase-positive staphylococci. The majority of *Staphylococcus* isolates showed

biofilm formation (75.1%) and slime formation (68.4%). The frequency of positive protease and lipase production for *S. aureus* isolates were 23.5 and 11.8%, respectively. Meanwhile, proteolytic and lipolytic activitiy were not found in the other *Staphylococcus* species. Fowoyo & Ogunbanwo (2016) investigated 225 CNS isolates for virulence traits and the standard and genotypic analysis revealed the production of biofilm in 200 isolates (78.4%), α-haemolysin in 136 (53.3%), β-haemolysin in 43 (16.9%), DNase in 199 (78.0%), TNase in 29 (11.4%), hyaluronidase in 125 (49.0%).

With the development of molecular biology, some techniques have been proposed for the detection of the genes responsible for toxins production. At present, Polymerase chain reaction (PCR) is one of the most frequently used methods as it enables the identification of the genes responsible for enterotoxin production with high sensitivity and specificity. Protocols for gene detection have been developed by using the sequence of oligonucleotides obtained by the computerized analysis of gene sequences (Jarraud, 1999). When investigating samples of CNS and *S. aureus* isolated from different foods in French restaurants, Rosec & Gigaud (2002) observed that none of the 74 isolated CNS samples contained toxigenic genes whereas, of the 258 *S. aureus* samples tested, 76 showed toxin genes. In Brazil, when investigating the presence of toxigenic genes in CNS samples isolated from food by the PCR technique, Cunha et al. (2006) found 10% of samples positive, of which 25% were positive for *sec*-1 gene and 75% for *sea* gene.

In the present study, the eight selected MDR strains were examined for presence of target enterotoxin genes namely; *ent*A, *ent*$D_{1,}$ *hl*g and *ent*C_1 using the total bacterial DNA isolated from the eight MDR strains as a PCR template. The resulting amplicons of PCR using designed specific primers, previously mentioned in materials and methods, are marked with arrows in Fig. 1 and 2. Incidence of target genes in tested strains was strain dependent and both *S. saprophyticus* 7 and *S. xylosus* 11 strains showed obvious significant amplicons of toxins genes *ent*A, *ent*D1 and *hlg* in addition to presence of *entC1* in *S. xylosus* 11 strain only. On the other hand, the clinical strains *S. aureus* 41, *S. epidermidis* 60 and *S. aureus* 66 possessed *ent*A and *hlg* genes only. Further amplification of toxins genes (*ent*A, *ent*C1, *ent*D1 and *hlg*) in *S. xylosus* 11 and *S. aureus* 41 strains was performed (Fig. 3 and 4). Isolation and sequencing of amplified genes and their alignment with database in Genbank confirmed similarity of *ent*A gene in both isolates *S. xylosus* 11 and *S. aureus* 41 to that of *S. aureus* strain ROF2 with accession JN687471.1 and AY827552.1, respectively. Meanwhile, *ent*D1 gene in *S. xylosus* 11 was similar to that of *S. xylosus* strain S170 with accession CP013922.1. *hlg* gene amplified from *S. xylosus* 11 was similar to that of *S. xylosus* strain S170 with accession CP013922.1. Moreover, Genbank alignment of amplified gene of *ent*C_1 in *S. xylosus* 11 showed similarity to *S. aureus* strain HUV05-03 with accession CP007679.1. All the alignment and similarities of the desired genes with Genbank sequences are shown in Table 4.

TABLE 3. Screening of the selected MDR isolates for production of certain virulence factors.

Test \ Isolate No	Clinical specimens						Food sources	
	SA41	SA66	SE60	SS61	Ssp.67	Ssp.69	SS7	SX11
Lecithinase	+	+	+	+	+	+	+	-
Coagulase	+	+	-	-	-	-	-	-
Gelatinase	-	-	-	-	-	-	-	-
Hemolysis	+	+	+	+	-	+	-	-
Protease	+	+	+	-	-	-	-	-
Amylase	+	+	-	-	-	-	-	-
Biofilm formation	+	+	-	+	-	-	+	+
Fibrinolytic activities	+	+	-	-	-	-	-	-

S. aureus 41=SA41, *S. aureus*=SA66, *S. epidermidis* 60=SE60, *S. saprophyticus* 61=SS61, *Staphylococcus* sp.67=Ssp.67, *Staphylococcus* sp.69=Ssp.69, *S. saprophyticus* 7=SS7, *S. xylosus* 11=SX11

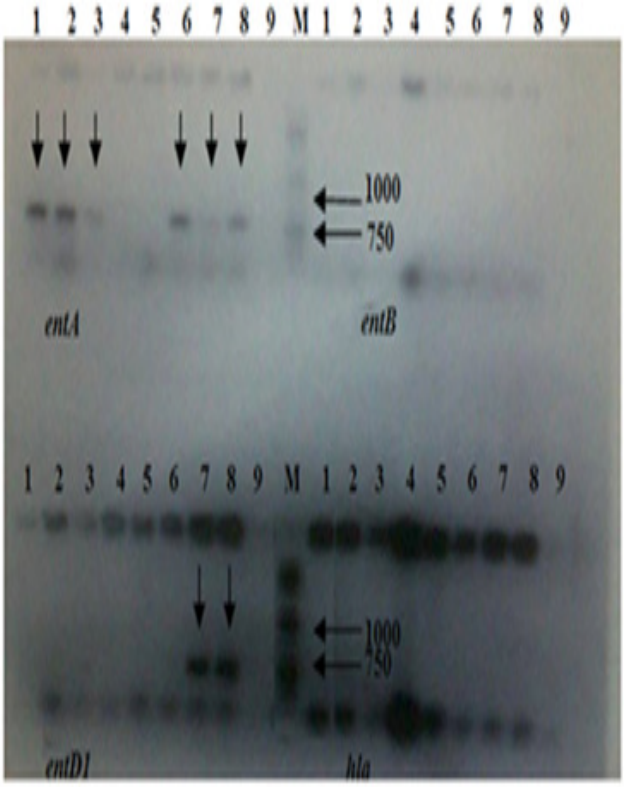

Fig. 1. Photo of agarose gel showing PCR products using specific primers for toxin target genes: *ent*A and *ent*D1 in the eight tested bacterial strains.

Lane M: 1k bp DNA marker. Lanes 1, 2, 3, 4, 5, 6, 7 and 8 are loaded with products of bacterial strains; *S. aureus* 41, *S. aureus* 66, *S.epidermidis* 60, *S. saprophyticus* 61, *Staphylococcus* sp. 67 and *Staphylococcus* sp. 69, *S. saprophyticus* 7 and *S. xylosus* 11, respectively and lane 9 is negative control. The resulting amplicons having molecular size 774 bp show presence of gene *ent*A in strains *S. aureus* 41, *S. aureus* 66, *S. epidermidis* 60, *Staphylococcus* sp. 69, *S. saprophyticus* 7 and *S. xylosus* 11, gene *ent*D1 with molecular size 777bp present in *S. saprophyticus* 7 and *S. xylosus* 11 strains.

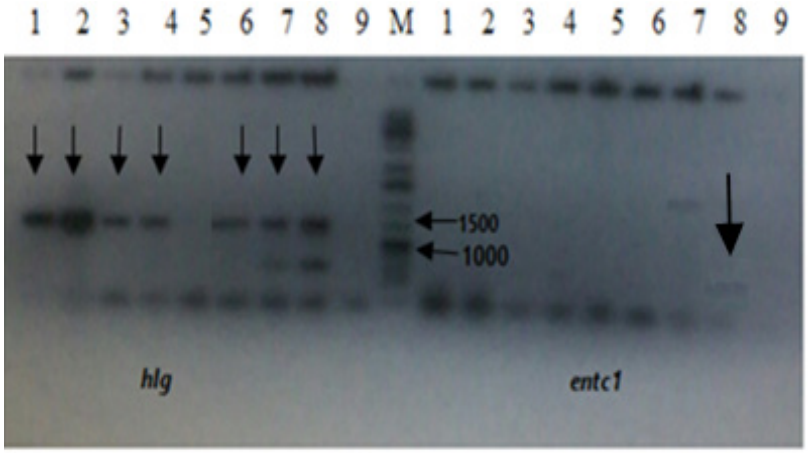

Fig. 2. Photo of agarose gel showing PCR products using specific primers for toxins target genes: *hlg* and *ent*C1 in the eight tested bacterial strains.

Lane M: 1k bp plus DNA ladder. Lanes 1, 2, 3, 4, 5, 6, 7 and 8 are loaded with products of bacterial strains; *S. aureus* 41, *S. aureus* 66, *S. epidermidis* 60, *S. saprophyticus* 61, *Staphylococcus* sp. 67 and *Staphylococcus* sp. 69, *S. saprophyticus* 7 and *S. xylosus* 11, respectively and lane 9 is negative control. The resulting amplicons show presence of gene *hlg* with molecular size 1200 bp, in strains *S. aureus* 41, *S. aureus* 66, *S. epidermidis* 60, *Staphylococcus* sp. 69, *S. saprophyticus* 7 and *S. xylosus* 11. Gene *ent*C1 (801 bp) was detected only in *S. xylosus* 11 strain.

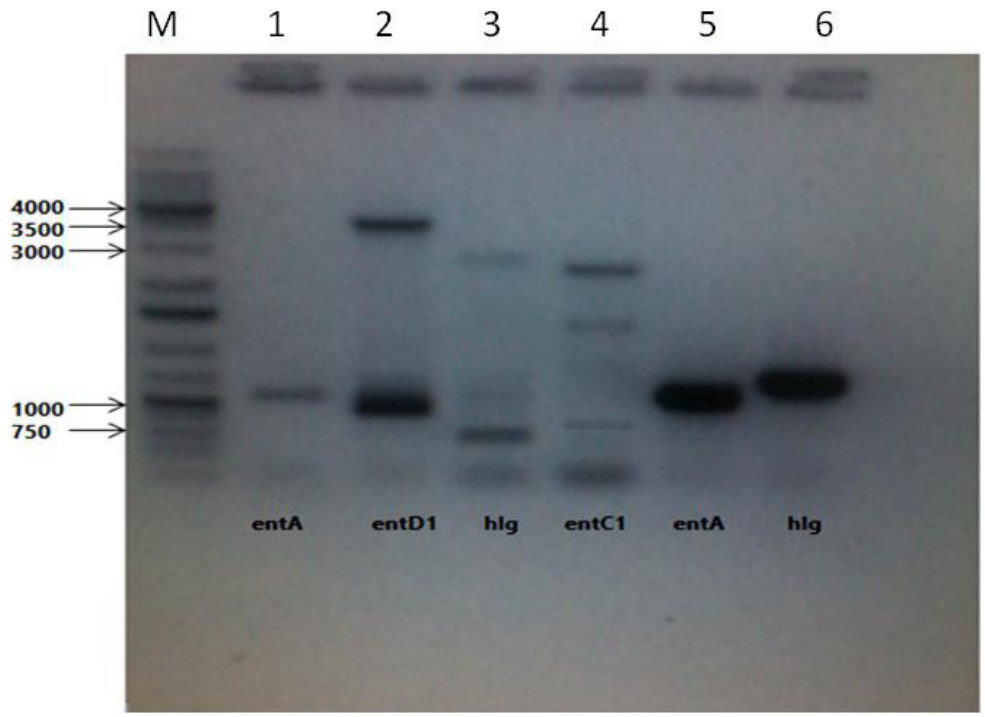

Fig. 3. Photo of agarose gel showing amplicons of PCR product resulting from 35 PCR cycles, using four mixtures for *S. xylosus* 11 strain (isolated from beef).

Lane1 (*ent*A), lane2 (*ent*D1), lane3 (*hlg*), and lane4 (*ent*C1), and using two mixtures for *S. aureus* 41 (isolated from pus); lane5 (*ent*A), and lane 6 (*hlg*). Lane M: 1k bp plus DNA ladder marker.

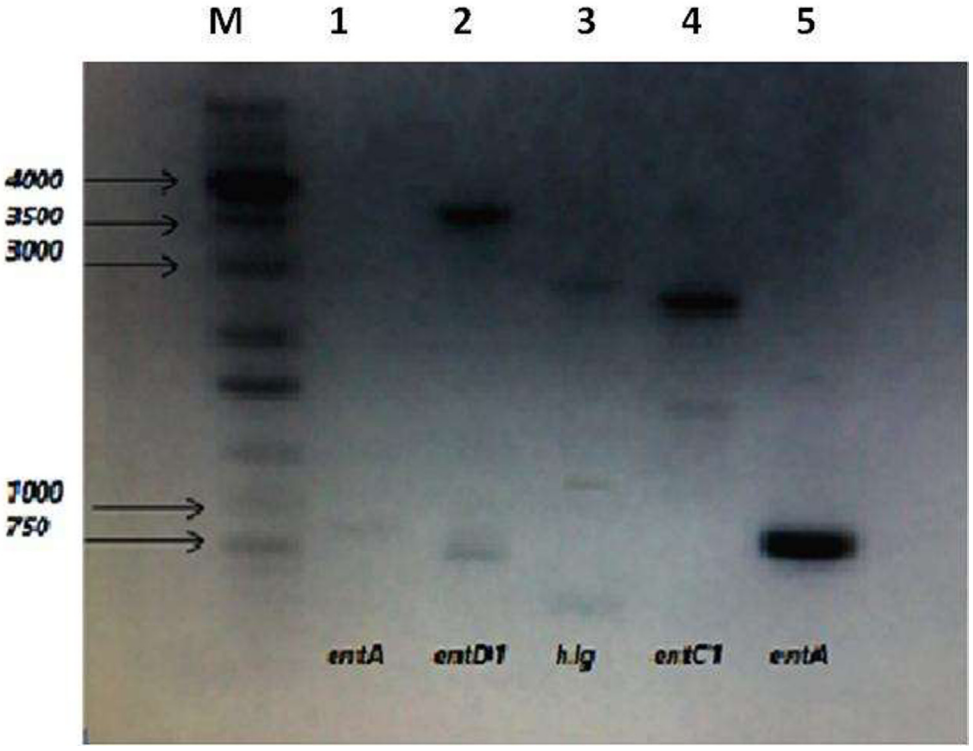

Fig. 4. Photo of agarose gel showing products of PCR after purification obtained amplicons. Four mixtures of the specific primers were used for *S. xylosus* 11 strain.

Lane1 (*ent*A), lane2 (*ent*D1), lane3 (*hlg*), and lane4 (*ent*C1) and using one mixture for *S. aureus* 41; lane 5 (*ent*A). Lane M: 1k bp DNA ladder marker.

TABLE 4. Similarities between the obtained sequences from *S. xylosus* 11 and *S. aureus* 41 and gene sequences in the database

Isolate	Target gene	Similar to	Similarity percentage	Accession number
S. xylosus 11	*Ent A*	*Staphylococcus aureus* strain ROF2 enterotoxin A gene, partial cds	79%	JN687471.1
	Ent D_1	*Staphylococcus xylosus* strain S170, complete genome	99%	CP013922.1
	Hlg	*Staphylococcus xylosus* strain S170, complete genome	99%	CP013922.1
	Ent C_1	*Staphylococcus aureus* strain HUV05 plasmid pHUV05-03, complete sequence	81%	CP007679.1
S. aureus 41	*Ent A*	*S.aureus* enterotoxin A (entA) gene, complete cds	99%	AY827552.1

Alignment and similarity percentages were obtained by blast program (https://blast.ncbi.nlm.nih.gov/Blast).

Many of the genes responsible for virulence factors are located in strain-specific genetic elements such as plasmids, transposons, bacteriophages (Baba et al., 2002). The association of enterotoxin genes with movable genetic elements results in the horizontal transfer of superantigen genes between staphylococcal lineages, and they constitute an important role in the evolution of *S. aureus* and of CNS as pathogens (Vasconcelos & da Cunha 2010).

Several authors have suggested the presence of enterotoxin and enterotoxin-like genes in genomes of *S. xylosus, S. chromogenes, S. saprophyticus, S. lentus, S. warneri, S. sciuri and S. haemolyticus, S. hyicus, S. simulans, S. epidermidis, S. succinus, S. capitis*, whereas the tst-1 gene was detected in *S. xylosus, S. saprophyticus, S. warneri, S. hominis,* and *S. haemolyticus* (Park et al., 2011). Fijałkowski et al. (2014) reported that among 30 isolates of *S. xylosus* isolated from milk, 16 (53.3%) harbored from 1 to 10 SAg genes. In total, in 16 SAg positive *S. xylosus*, 11 different enterotoxin genes were detected: *sec, sed, seg, seh, sei, selm, seln, selo, selp, ser, selu* and one *etd* gene encoding exfoliative toxin D. The most prevalent genes were *ser, selu* and *selo*. Among all the positive isolates of *S. xylosus*, a total of 14 different SAg gene combinations were detected. One combination was repeated in 3 isolates, whereas the rest were detected only once. Piechota et al. (2014) detected staphylococci enterotoxin genes in *Staphylococcus* species isolated from milk samples from which 22 (13.7%) were coagulase-negative staphylococci (CNS), among them in 9 (11.4%) isolates of *S. xylosus*, 5 (16.7%) *S. sciuri*, 3 (10.3%) *S. epidermidis* and in 5 (22.7%) *Staphylococcus* spp. In some CNS 2 or 3 genes were detected simultaneously. Among the investigated enterotoxin genes, *sec* was the most prevalent (70%). The genes encoding enterotoxin B and D were detected in 5 (16.7%) and 6 (20%) isolates, respectively. The lowest number of isolates had *sea* and *see* genes. Nunes et al. (2015) reported the presence of *S. saprophyticus, S. sciuri, S. xylosus, S. carnosus, S. succinus, S. epidermidis,* and *S. hominis* in commercial and artisanal salami collected in the municipality of Rio de Janeiro, Brazil. Fifteen strains harbored multiple enterotoxin genes, with high incidence of *seb/sec* and *sea* (57% and 50%), respectively; intermediate incidence of *sed/seh/selm* and *sei/seln/tst*-H (33% and 27%), respectively and low incidence of *see/selj/selo* and *seg,* (13% and 1%). Fowoyo & Ogunbanwo (2016) isolated 255 CNS isolates from 6 traditional fermented food from North Central Nigeria. These isolates were identified as *S. epidermidis*, *S. simulans*, *S. xylosus*, *S. kloosii*, and *S. caprae*, CNS isolates producing enterotoxins SEA, SEB, SEC, and SED were detected in 61 (23.9%), 19 (7.5%), 9 (3.5%), and 8 (3.1%) of examined food samples, respectively.

Identity of the selected strain *S. xylosus* 11 was confirmed by amplification and alignment of 16S rRNA gene which showed 99% similarity with *S. xylosus* strain and submitted in Genbank under accession number MH118574 (Fig. 5).

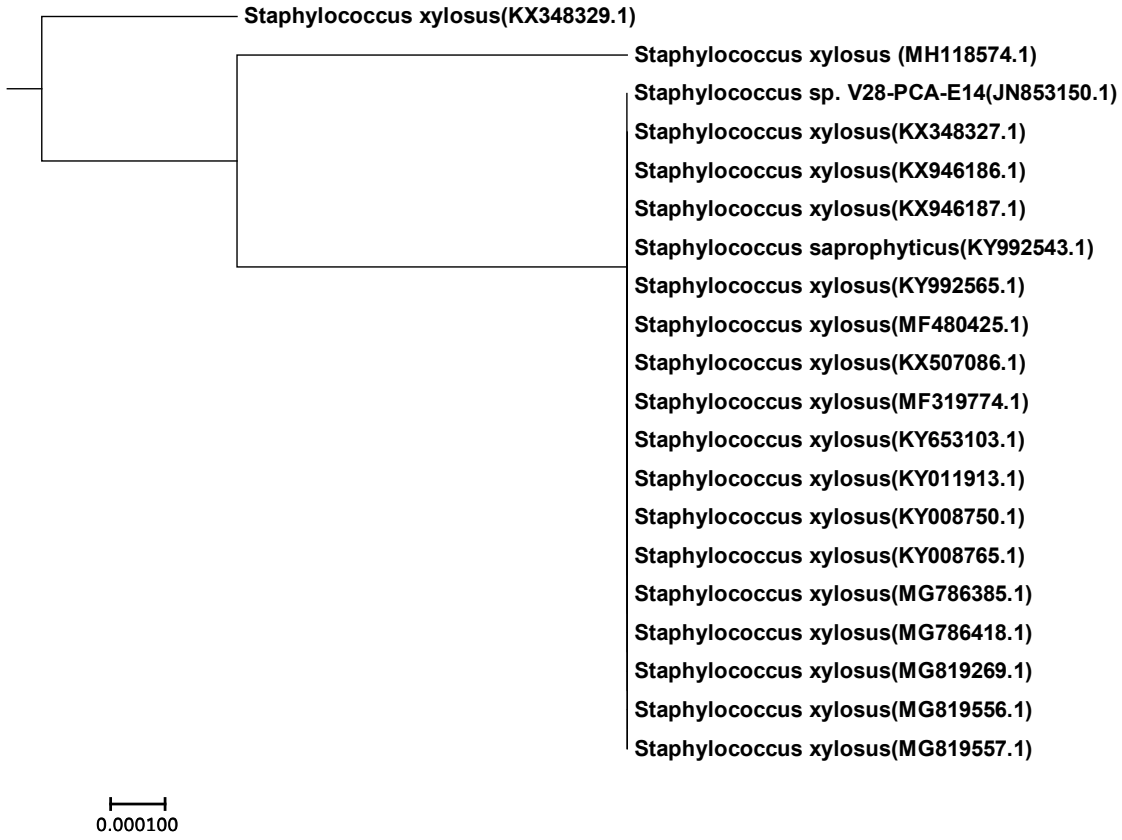

Fig. 5. Phylogenetic tree of *Staphylococcus xylosus* 11.

Staphylococcus xylosus is a commensal of the skin of humans and animals persists in soils and on surfaces (Shale et al., 2005) and can be found in various niches (Nimrat et al., 2006). It is a ubiquitous bacterium naturally present in food and is one of the major starter cultures used for meat fermentation. Meanwhile, few strains could potentially be hazardous and are related to animal opportunistic infections (Dordet-Frisoni et al., 2007). *S. xylosus* in addition to its ability to form biofilms (Planchon et al., 2006), the ubiquity of *S. xylosus* might be explained by its ability to adapt to different environments. Also, *S. xylosus* had been previously reported as one of the most prevalent CNS species involved in bovine intramammary infections contaminating produced milk (Feßler et al., 2010) and one with the highest percentage of genes encoding enterotoxins (Piechota et al., 2014 and Nunes et al. 2015). However, distinguishing clinically significant pathogenic strains from those that are only sample contaminants is one of the greatest problems faced by clinical laboratories (Cunha et al., 2006).

Conclusion

Various studies featured *Staphylococci* as important pathogens and pointed out their toxigenic potential confirming that greater attention had to be given to such microorganisms, which are still often considered by other researchers to be simply contaminants.

Further investigation on environmental factors and elucidation of the regulation mechanisms interfering with virulent genes expression in CNS must be evaluated as well. The ability of *Staphylococci* food model strains to produce enterotoxins and their drug multi-resistance character must be examined when evaluating the safety hazards of food poisoning. Safety measures should be taken to reduce or totally remove the occurrence of *Staphylococci* in foods.

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انتشار عوامل الضراوة في المكورات العنقودية المعزولة من العينات السريرية والأطعمة في مصر

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تم عزل 100 عزلة من المكورات العنقودية موجبة جرام من 80 عينة سريرية و 20 عينة غذائية باستخدام وسائط بيرد باركر و أجارملح مانيتول. أثبتت النتائج أن 60 عزلة من أصل 80 (75%) معزولة من العينات السريرية تنتمى إلى المكورات العنقودية الإيجابية لاختبار تخثر الدم في حين أن 20 عزلة أخرى (25%) تنتمى إلى المكورات العنقودية السالبة لاختبار تخثر الدم، كما أظهرت النتائج أن جميع المكورات العنقودية المعزولة من عينات الطعام (20 عزلة) سالبة لإختبار تخثر الدم. باختبار حساسية هذه العزلات تجاه 11 نوع مختلف من المضادات الحيوية تبين أن 8 عزلات منها مقاومة لأكثر من 4 مضادات حيوية، وتم تعريف هذه العزلات عن طريق صفاتها الشكلية والفسيولوجية والأختبارات البيوكيميائية. تم تعريف العزلات الإكلينيكية الإيجابية لاختبار تخثر الدم وعددهم اثنان (41 و 66) بشكل مبدئي على أنها بكتريا *S. aureus* و كذلك تم تعريف 6 عزلات عنقودية سالبة لإختبار تخثر الدم من جنس المكورات العنقودية *Staphylococcus*، تم فحص بعض الجينات المسئولة عن انتاج السموم المعويه وهي: *ent*A، *ent*C، *ent*D1، *hlg* و في 8 من العزلات المختارة و المقاومة لعديد من المضادات الحيوية. أظهرت النتائج وجود الجينات *hlg* و *ent*D1 و*ent*A فى العزلات رقم 7 و 11 بينما وجدت الجينات *ent*A و *hlg* فقط فى العزلات 41 و 66، تم تعريف هذه العزلات مبدئيًا على أنها *S. saprophyticus* 7 و *S. xylosus* 11 و العزلات السريرية 41 و 66 على أنها *S. aureus*. تم تأكيد تعريف *S. sylosus* 11 التي تحتوي على أكثر من 3 جينات سمية باستخدام 16S rRNA التي أظهرت تشابه 99% مع سلالة *S. xylosus* تم تسجيل هذا التسلسل في بنك الجينات تحت رقم MH118574.